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Surface cAMP Receptors Mediate Multiple Responses during Development in *Dictyostelium*: Evidenced by Antisense Mutagenesis

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Abstract. Cell surface cAMP receptors (cARs) have been implicated in multiple aspects of development in *Dictyostelium*. Antisense mutagenesis has recently provided strong evidence that cARs are necessary for aggregation (Klein et al., 1988. *Science (Wash. DC)*. 241:1467-1472). We show here that the expression of cAR1 antisense mRNA which prevents the appearance of cAR1 antigen also prevents the expression of cAMP-binding activity and blocks multiple cAMP-mediated responses. Chemotactic sensitivity to cAMP was lost as were stimulus-induced cAMP and cGMP production. Furthermore, the expression of developmentally regulated marker genes, dependent on repeated cAMP stimulation, was altered. As a result, the developmental program was severely impaired; most of the cells failed to aggregate and undergo further differentiation.

During development *Dictyostelium* amebae synchronously aggregate into a multicellular organism, and cells in specific positions differentiate into stalk or spore cells (Janssens and Van Haastert, 1987; Gerisch, 1987). The aggregation of individual cells into the multicellular structure is coordinated by a developmentally regulated cAMP signaling system in which binding of cAMP to surface receptors triggers activation of adenylate cyclase. Newly synthesized cAMP is then secreted, completing a positive feedback loop which, coupled to rapid, reversible adaptation of the response, causes cAMP levels to oscillate. The oscillatory signal is relayed to distal cells and the chemotactically sensitive cells aggregate in response to the propagated cAMP waves (Janssens and van Haastert, 1987). The cAMP oscillations are also required for early gene expression which is suppressed by a constant level of extracellular cAMP. In contrast, late gene expression requires constant extracellular cAMP (Gerisch, 1987; Schaap and van Driel, 1985; Oyama and Blumberg, 1986; Haribabu and Dottin, 1986; Kimmel, 1987; Mann and Firtel, 1987). Mutants with defective oscillators fail to differentiate, but can be rescued by periodic, followed by constant, application of exogenous cAMP.

We have recently discovered a cell surface cAMP receptor, designated cAR1.[1] This receptor is anticipated to mediate many cAMP-induced responses in early development (Theibert and Devreotes, 1986; Van Haastert, 1984*a*). Stimulation with cAMP also induces a rapid fivefold increase in phosphorylation of cAR1. The kinetics and dose dependence of phosphorylation parallel those of the adaptation, suggesting that phosphorylation of cAR1 plays a central role in adaptation (Klein et al., 1985; Devreotes and Sherring, 1985; Vaughan and Devreotes, 1988).

The application of antisense RNA or DNA sequences to block the expression of many eukaryotic genes has been reviewed (Stout and Caskey, 1987; van der Krol et al., 1988); and in *Dictyostelium*, interference in the expression of discoidin (Crowley et al., 1985) and myosin heavy chain (Knecht and Loomis, 1987) have been reported. We have previously reported that a cAR1 antisense cell line failed to express cAR1 antigen or aggregate. In this report we characterize a number of cAR1 antisense cell lines. We have found that cAMP binding sites are not expressed and a multitude of receptor functions are lost, demonstrating the central role of cAR1 in development.

Materials and Methods

Antisense Constructs and Transformation

In addition to the previously described construct, (Klein et al., 1988) which uses the actin 6 (A6) promoter, two other constructs were also designed. cDNA of cAR1 was inserted into the Bgl II site of vector BS18 (a gift from Richard Firtel, Department of Biology, University of California at San Diego) which uses an actin 15 promoter to generate complementary RNA in the transformed cell. For the third construct, a 1.9-kb fragment derived from the 5′ region of a cAR1 genomic clone was inserted in the Xba I site of p6B (cAR1 cDNA in Bluescript, see Klein et al., 1988) and cotransformed with BS18. Since cells transformed with this genomic clone did overexpress cAR1 with a developmental pattern similar to wild type (our unpublished observation), it is likely the upstream fragment contains the cAR1 promoter. Cells (strain AX-3) were transformed with these vectors according to Knecht and Loomis, 1987. During prolonged passage of some antisense cell lines, efficiency of antisense decreased as evidenced by a shortening in the duration of the aggregation block. This effect appears to be due to the loss of copies of the integrated vector. Although amebae can usually

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1. *Abbreviation used in this paper*: cAR, cell surface cAMP receptor.

be stored in liquid nitrogen, this process somehow interferes with the retention of the phenotype of antisensed cells. Therefore, new transformations were performed every 3 mo.

In analyses of genomic DNA, using the full-length cAR1 cDNA as a probe, both the control and the antisense transformants had a 3.5-kb Xba I fragment and a 12.9-kb Eco RV fragment (data not shown). Because these fragments are identical to those of the parental cell line, AX-3, we concluded that the antisense vector did not integrate into the cAR1 gene by homologous recombination. In the antisense transformants a second darker band was observed in both restriction digests. Based on the size of this band (7.8 kb) and the restriction enzyme used (single site in the vector), we concluded that the majority of the vector integrated in tandem repeats of up to 50 copies.

Development of Cells

Cells were allowed to develop in shaken suspension (100 rpm) in DB (5 mM Na_2HPO_4, 5 mM KH_2PO_4, 2 mM $MgSO_4$, 0.2 mM $CaCl_2$) at 2×10^7 cells/ml, or on the surface of starvation plates (DB, 1% agar) at 6.4×10^5 cell/cm^2. Cells developed on starvation plates were photographed as described (Gross et al., 1976).

RNA, DNA Preparation, and Analysis

Total RNA of developed cells was prepared as described (Klein et al., 1988). Approximately 10 μg of RNA per lane were electrophoresed in 1.2% formaldehyde agarose gels and blotted to nitrocellulose (Maniatis et al., 1982). Total DNA was prepared by a modification of the DNA minipreps (Nellen et al., 1987), then digested with restriction enzyme overnight, electrophoresed in 0.7% agarose gel, and blotted to nitrocellulose. The nitrocellulose was baked in an 80°C vacuum oven for 2 h, and hybridized with specified probes. RNA probes were prepared as suggested by the manufacturer (Promega Biotec, Madison, WI). DNA probes were made by the random priming method (Feinberg and Vogelstein, 1983) from fragments isolated from agarose gels.

Immunoblot

Total protein of developing cells was prepared by lysis directly into sample buffer (10% glycerol, 5% DTT, 3% SDS, 6.25 mM Tris, pH 6.8, 0.25% Bromophenol blue) at a final density of 5×10^6/ml. Extracts were subjected to SDS-PAGE and transferred to nitrocellulose. Polyclonal rabbit antisera against cAR1 (Klein et al., 1988) and ^{125}I-protein A were used to detect the receptor protein.

cAR1 Functional Assay

cAMP binding assays were carried out as previously described (van Haastert and Kien, 1983). Both ammonium sulfate and phosphate buffer assays were used. cAMP-stimulated cAMP production was determined by an isotope dilution assay using 5×10^{-6} M 2′-deoxy-cAMP as stimulus in the presence of 5 mM DTT (Van Haastert, 1984*b*). The time course and amount of cGMP production was determined by a RIA (van Haastert and van der Heijden, 1983) after stimulation with 10^{-7} M cAMP.

Chemotaxis

Cells were developed for 3–7 h and then washed off the agar and used in the small population assay to determine chemotaxis responsiveness towards cAMP (Konijn, 1970).

Results

Characterization of Antisense Cell Lines: Absence of [^{3}H]cAMP Binding Activity

As previously reported, control and antisense transformed cell lines displayed dramatically different phenotypes. The controls underwent normal development; characteristic waves

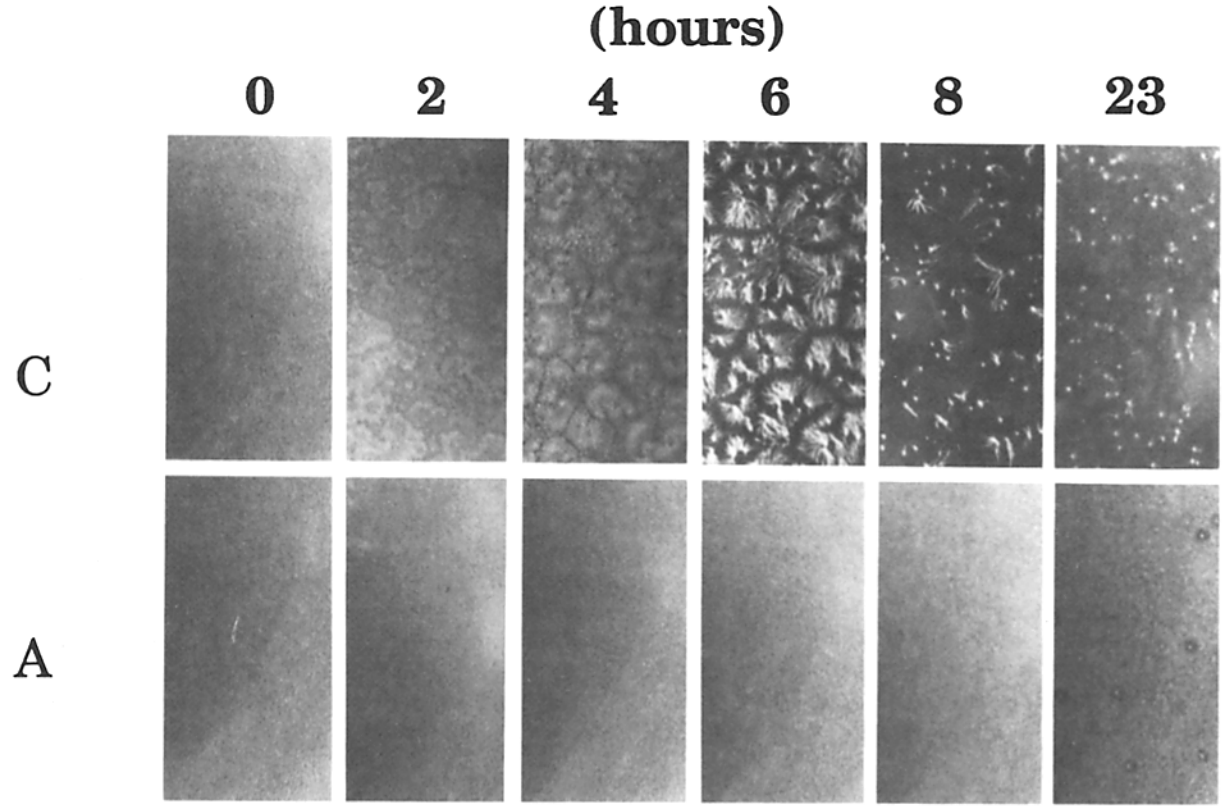

Figure 1. Antisense cell lines fail to aggregate. Cell lines stably transformed with cAR1 antisense constructs or control vectors derived from vector A6, were grown in HL5 medium supplemented with 20 μg/ml of G418 and 30 μg/ml of dihydrostreptomycin. Amebae were harvested and washed in DB and plated at 5×10^7 cells per dish (100 mm) of DB-agar. Plates were incubated at 22°C. Control and antisense transformants are shown in row *C* and *A*, respectively. The hours of development are indicated. Each panel shows a 2 × 5 cm portion of the monolayer.

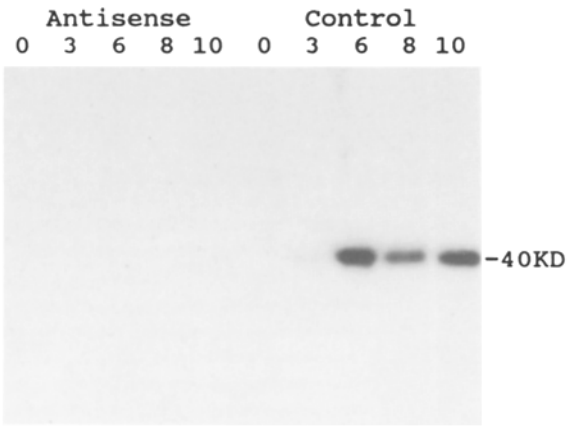

Figure 2. Absence of cAR1 antigen in antisense cell lines. Cells were transformed with either vector BS18 or the antisense construct derived from vector BS18. Individual clones were developed on the starvation plates and harvested at 0, 3, 6, 8, or 10 h. Extracts of 5×10^6 cells were immunoblotted with specific cAR1 antiserum as described under Materials and Methods.

and streams were apparent within a few hours after starvation. The multicellular stages of development such as mounds, fingers, slugs, Mexican hats, and fruiting bodies (at 24–26 h) appeared normal (Fig. 1). During the same time period the antisense transformed cell lines remained as uniform monolayer. By 23 h, a small fraction (<5%) of the cells entered into tiny aggregates which did not form streams, but eventually gave rise to fingers and slugs, and small fruiting bodies (Fig. 1). The remaining cells had not aggregated after two weeks.

As reported previously, normal expression of cAR1 antigen (mol wt, 40,000) was detected in immunoblots of the control transformed cells; it rose from undetectable levels in growing cells to a maximum during the aggregation phase of development. In contrast, there was no detectable cAR1 antigen in the antisense cell lines at any time (Fig. 2). We found that the [^{3}H]cAMP binding activity of the control transformants increased in parallel to the expression of cAR1 antigen reaching a maximum of $\sim 10^5$ sites/cell at 6–8 h of development; however, the [^{3}H]cAMP binding activity of antisense transformants remained close to the basal level throughout the developmental program (Fig. 3).

We also examined the mRNA in the antisense transformants. The control transformants showed a developmentally regulated expression of cAR1 mRNA which, similar to wild type, peaked at 3 h, declined until 9 h, and increased slightly at 12 h (Fig. 4 *b*, *bottom*). The mechanism by which the antisense vectors, A6 and BS18, inhibit production of cAR1

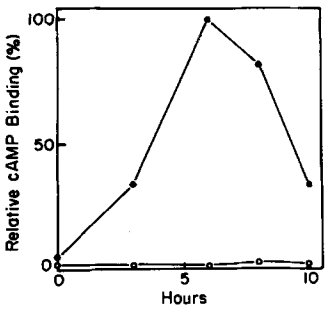

Figure 3. [^{3}H]cAMP binding in transformed cell lines. Constructs were those derived from vector A6. Cells were developed in suspension at 5×10^7 cell/ml in DB. All binding data were normalized to peak binding of the control transformants.

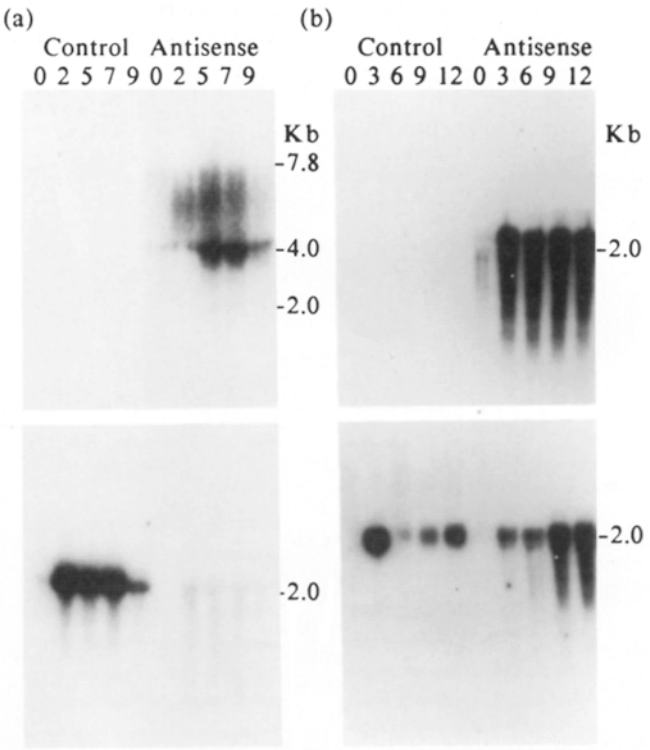

Figure 4. Northern analysis in the antisense and control transformants. (*a*) Cells transformed with A6 vector were developed in shaken suspension at 5×10^7 cell/ml. (*b*) Cells transformed with BS18 vector were developed on starvation plates. Total RNA was prepared at the specified times (in hours). Sense and complementary RNA probes were transcribed from linearized p6B (Klein et al., 1988) with T3 or T7 polymerase in the presence of [α-^{32}P]-UTP. Top panels were hybridized with the sense probes and the bottom panels with the complementary probe. Note that the A6 construct transformants contained antisense RNA which included two major bands at 7.8 and 4.0 kb (Fig. 4 *a*, *top*). The 7.8-kb band is the expected size of the transcript, while the 4-kb band is probably due to fortuitous termination within the 5′ end of cAR1 cDNA. The BS18 construct cells have abundant antisense RNA with a major band at 2 kb and smaller fragments (Fig. 4 *b*, *top*).

protein may differ. In antisense transformants made from the vector A6 construct, there was a negligible amount of endogenous cAR1 mRNA (Fig. 4 *a*, *bottom*), while in those made from the vector BS18 construct, there was a considerable amount of endogenous cAR1 mRNA present (Fig. 4 *b*, *bottom*), although we did not determine its integrity. In spite of these differences in RNA blots, both types of constructs led to a complete loss in cAR1 antigen and [^{3}H]cAMP binding activity as exemplified in Figs. 2 and 3.

Antisense Mutagenesis Arrests the Developmental Program by Inhibiting Multiple Responses

Binding of cAMP to surface receptors triggers activation of internal adenylate and guanylate cyclases leading to increased accumulation of cAMP and cGMP (Theibert and Devreotes, 1986; van Haastert and van der Heijden, 1983). When control transformants were stimulated, cAMP levels increased from undetectable levels to ~100 pmol/10^7 cells within 2 min (Fig. 5 *a*). cGMP levels rose from 5 pmol to a peak of 25 pmol/10^7 cells within 10 s, and returned to the basal levels by 50 s (Fig. 5 *b*). In contrast, similarly stimu-

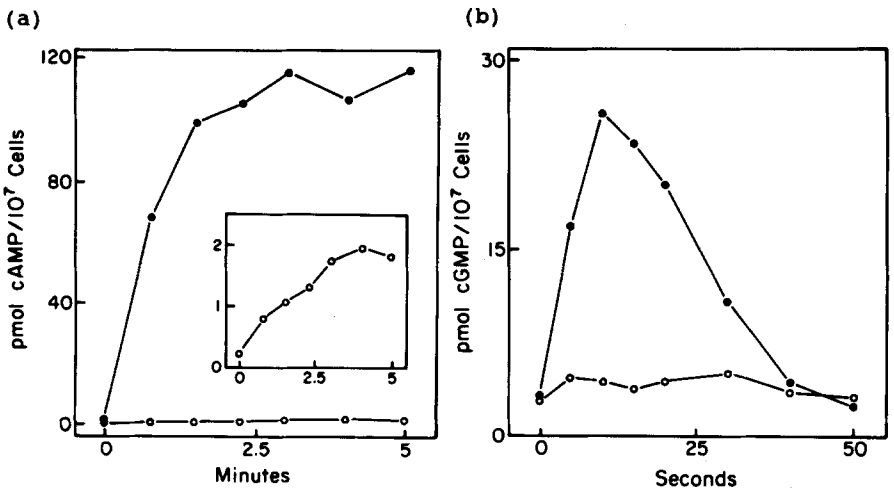

Figure 5. Failure to synthesize cAMP and cGMP. Transformed cells were developed in DB for 5 h and the stimulus-induced (*a*) cAMP production was assayed by isotope dilution, and (*b*) cGMP production was assessed by RIA. (○) Antisense transformants; (●) control transformants. cAMP levels do not fall since it is rapidly secreted into the medium which contains phosphodiesterase inhibitors.

lated antisense transformants displayed only marginal increases in cAMP production to 2 pmol/10^7 cells (Fig. 5 *a*, *inset*); cGMP levels did not increase (Fig. 5 *b*).

The chemotactic responses of growing cells toward folic acid and of early developmental stage cells toward cAMP have been well documented (26, 27). The role of cAR1 in chemotaxis was assessed in the antisense transformants. Cells starved on agar for 7 h were assayed by the "small population" method. The control transformants began to show positive responses with 10^{-9}–10^{-8} M cAMP; 100% of the populations responded to 10^{-7} M (Fig. 6). In contrast, the antisense transformants did not respond to concentrations of cAMP below 10^{-6} M, and only 10% of the populations responded at the highest doses (Fig. 6). However, when folic acid was tested as a chemoattractant for undifferentiated cells, both control and antisense transformants responded identically (data not shown). Thus, the chemotactic response to cAMP is specifically impaired in the antisense transformants.

The expression of several marker genes was compared in the control and antisense cell lines. The absence of cAMP receptors greatly reduced the cAMP-induced expression of the gene D2 (Fig. 7 *a*), a serine esterase, normally induced during early development, and completely prevented the expression of the spore coat proteins sp96, sp70, and sp60 (Fosnaugh and Loomis, 1990). Surprisingly, the major Gα2 mRNA which, in control cells, appears in parallel with that of cAR1, increased normally in the antisense cells during the first 3 h. However, its level plateaued by 12 h and did not decrease as it did in the control cells (Fig. 7 *b*).

Discussion

Our characterization of the antisense transformed cell lines demonstrates the essential role of cAMP receptors in the developmental program. The presence of complementary RNA in these cell lines inhibited the expression of cAR1 and resulted in a characteristic phenotype. The cells displayed severely retarded aggregation, negligible cAMP binding, little ligand-induced cAMP or cGMP accumulation, very weak chemotactic responses, and failure to express or properly regulate a number of developmental marker genes.

During starvation, the control cells developed cAMP binding activity in parallel with the appearance of cAR1 protein (mol wt, 40,000) as detected by the cAR1 antisera in immunoblots. Under the same conditions, antisense cells displayed very low cAMP binding activity and no observable cAR1 protein. Proper developmental expression of cAR1 mRNA (2 kb) was observed in control transformants. In the A6 vector construct transformants, only weak hybridization to fragments smaller than 2 kb was detected. Although a considerable amount of apparent 2-kb mRNA persisted in the cell lines transformed with the BS18 vector construct, the absence of cAR1 protein showed that it was not translated. These observations suggest that antisense mutagenesis may block production of the protein either by destabilizing the mRNA or by preventing its translation. The analysis of genomic DNA showed identically sized cAR1 bands in both the antisense and the wild-type cells in several digests, suggesting that gene disruption did not occur in the antisense transformants, and that the phenotype is most likely due to the presence of antisense RNA. Since our major goal was to elucidate the role of cAR1 in development, we have not pursued these mechanistic studies further.

Recent observations indicate that cAMP signals are transduced through cAR1 via the Gα2 protein (Kumagai et al., 1989; Pupillo et al., 1989). The time of peak production of cAR1 and the major Gα2 mRNA during development are nearly coincidental, suggesting that the components of the signal transduction pathway are coordinately expressed. We were surprised to find that induction of Gα2 does not appear to require expression of cAR1 at the cell surface, since its RNA levels increased normally in the antisense transformants. However, there was no decline in the antisense trans-

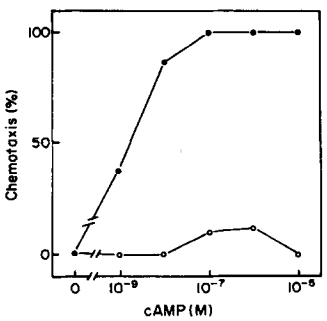

Figure 6. The inhibition of chemotaxis in antisensed cells. Cells starved on DB agar for 7 h were used to conduct the small population assay to determine the cAMP chemotaxis. (○) Antisense cells; (●) control transformants. Shown are the fraction of small populations responding.

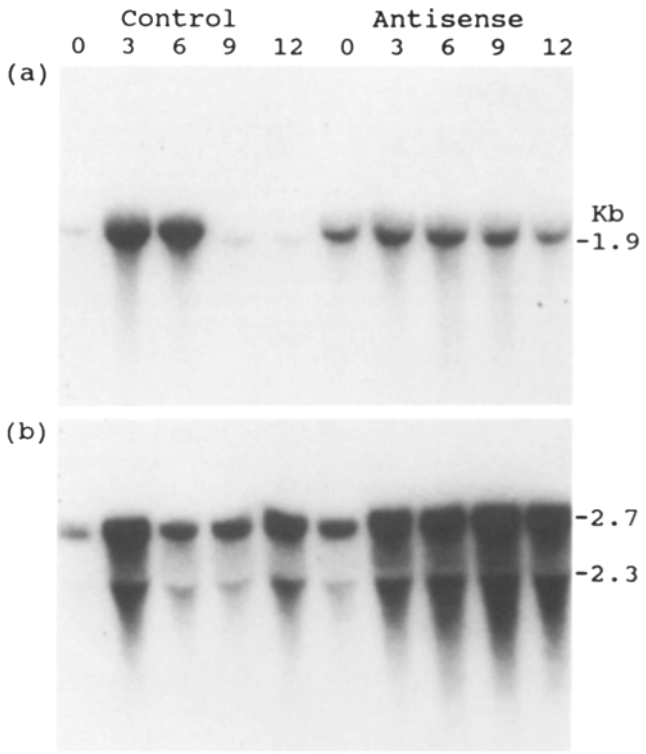

Figure 7. Expression of developmentally regulated marker genes. RNA blots were prepared as described in Fig. 4 *b* and probed with full-length cDNA (*a*) D2 (gift from Richard Firtel) or (*b*) Gα2.

formants suggesting that cAR1 may control the repression of Gα2, which occurs after aggregation.

When individual clones from a transformation were examined, the phenotypes of some appeared weaker or stronger than the mean. That is, in some clones there was a long delay before aggregation rather than a permanent block and a large percentage of the cells eventually differentiated. The phenotype of each clone was reproducible and those that were delayed least contained significant levels of cAR1 antigen. As noted above, even in the transformants with strong phenotypes a small fraction of the cells eventually aggregated. However, it was not possible to determine whether these few cells were expressing cAR1, and thus we cannot conclude whether the small aggregates require this receptor. This phenotype, in which cells within small territories aggregate without streams, may be of interest because it is similar to that of *D. minutum*, an evolutionarily related forerunner of *D. discoideum*. This species lacks the cAMP signaling system and early cAMP binding activity, but displays elevated cAMP binding during the later multicellular stages (Schaap et al., 1984).

Recent observations suggest that cAR1 is a member of a family of cARs that are differentially expressed during the developmental program. The functions of and the relationships between the individual cARs are not known at present, but it is reasonable to believe that each cAR has a distinct role in the development. Our studies indicate that cAR1 is required for a multitude of cAMP-mediated responses in early development, although we cannot exclude the possibility that related cARs, expressed later in development, are required for certain functions. Although the cAR genes cross-hybridize under relaxed conditions, it is not clear whether cAR1 antisense mRNA can prevent expression of other cARs in vivo. The clean block of responses to cAMP, without affecting those to folic acid, indicates that it does not interfere with expression of the folic acid receptor. Expression of another cAR may also be prevented because it may be dependent on cAR1. We are attempting to construct null mutants of the individual cARs by homologous recombination to address these important issues.

We would like to thank Richard Firtel for providing the BS18 vector and cDNA probe, D2; Ron Johnson for the construction of BS18 antisense vector, and contribution of the 5′ fragment cAR1; Fanja Kesbeke for the technical help; and Peggy Ford for the typing of this manuscript.

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